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Effects of Vinyl Chloride Exposures to Rats Pretreated with Phenobarbital

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Male rats were exposed to 10 consecutive days, 6 hr/day, to vinyl chloride vapors at an average concentration of 13,500 ppm. The exposed rats were divided into three groups of eight rats each: one group was pretreated with 3-methylcholanthrene, one group was pretreated with phenobarbital, and the third group received no treatment. Half the animals in each group were sacrificed 18 hr after the last exposure and half were sacrificed 4 days later. In a second experiment, four rats pretreated with phenobarbital were exposed to vinyl chloride vapors at a concentration of 17,300 ppm for 2 days and sacrificed about 9 A.M. on the third day. In both experiments control animals, also treated with phenobarbital or 3-methylcholanthrene, were exposed to air only. At the time of sacrifice, lungs, kidneys, spleen, heart, and a small piece of liver from each animal were preserved for histological examination. The remainder of the liver was processed for assay of microsomal enzyme activity.

The following parameters were investigated: growth rate, organ weights, morphological changes, and both benzphetamine-*N*-demethylase activity and cytochrome P-450 content of microsomes prepared from the livers. In both experiments the only marked difference noted in any group was a decrease in the growth rate of the animals exposed to vinyl chloride and treated with phenobarbital. This decreased growth rate was particularly apparent on the third day of the vinyl chloride exposures. Occasional morphological changes were also seen in the livers of the animals treated with phenobarbital and exposed to vinyl chloride.

Introduction

The recent discovery that vinyl chloride is a carcinogen (*1–3*) has prompted a renewed interest in this compound. Several groups are currently studying the mechanism whereby vinyl chloride causes tumors, and preliminary reports have appeared (*4,5*). This paper summarizes our studies of the acute toxicity of vinyl chloride as affected by two known inducers of hepatic microsomal xenobiotic metabolizing enzyme activity, 3-methylcholanthrene (3-MC) and phenobarbital (PB).

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Methods

Animals

Male rats (Charles River CD-1) weighing between 200 and 250 g were used in these studies. They were housed in plastic cages with corncob bedding. Food (Wayne Lab Blox) and water were provided *ad libitum* except during the inhalation exposures. A 12-hr on–12-hr off light cycle was used in the animal quarters.

Reagents

Vinyl chloride (Linde Specialty Gas) was verified to be greater than 99.5% pure by gas chromatography and was used as supplied.

HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were purchased from the Sigma Chemical Co. All other chemicals were reagent grade of commercial origin.

Exposures

A pair of 128-liter stainless steel–Lucite chambers enclosed in a separately exhausted plenum (*6*) were modified to provide a closed dynamic system. In addition to the chambers, the system included a circulation pump, a vacuum-tight vessel containing CO_2 absorber and a filter. To charge the system, the vacuum vessel was evacuated and then filled with vinyl chloride. The circulation system was then started, and the vinyl chloride continuously recirculated. Oxygen concentrations were monitored with a Bentley oxygen meter and oxygen was added to maintain a partial pressure of 140 torr. The system was shut down by exhausting the chamber atmosphere through a charcoal filter and then out the exhaust stack. Use of this recirculating system minimized the amount of vinyl chloride used and thus minimized the potential for escape into the environment. Control animals were exposed in a similar fashion without adding vinyl chloride. Exposures were for 6 hr/day.

The concentration of vinyl chloride in the exposure chamber was monitored at hourly intervals. Samples of chamber air were taken and bubbled into reagent grade benzene. After a 5-min equilibration, the amount of vinyl chloride in an aliquot of this benzene was determined by chromatographic analysis using a Varian Aerograph 1400 gas chromatograph equipped with a flame ionization detector. The 6 ft × ⅛ in. stainless steel column was packed with 3% SE 30 on 60/80 Gas-Chrom-P. Column temperature was 50°C, detector temperature was 225°C, and the flow rate of the nitrogen carrier gas was 15 ml/min. A standard curve of vinyl chloride in benzene was linear over the range of concentrations used. A time weighted average concentration of vinyl chloride in the exposure chamber was calculated for each day; the concentrations reported are the average of the daily time-weighted values.

Chemical Treatment of Animals

PB (1 mg/ml) was given in the drinking water starting 3 days before the first day of inhalation exposure and continuing up to 1 day after the last exposure. 3-MC was dissolved in cottonseed oil and injected IP at a dose of 15 mg/kg starting on the day before the first exposure. It was given on three consecutive days and on alternate days thereafter, the last dose being given just prior to the last exposure. Control animals were not treated.

Morphological Studies

Animals were killed by cervical dislocation, and a small piece of liver (ca. 1 mm^3) quickly diced in glutaraldehyde for electron microscopy. The remaining portion of the liver was weighed and a section was taken for light microscopy. The bulk of the liver was put into ice-cold 1.15% KCl–0.0013*M* HEPES buffer at pH 7.6 (KCl–HEPES) and held for subsequent biochemical assay. The lungs and trachea were removed intact, weighed, infused with 3–4 ml of 10% neutral buffered formaldehyde and stored in this fixative. Kidneys, spleen, and heart were also weighed and stored in formaldehyde.

Biochemical Studies

The microsomes were prepared from the livers by methods in use in our laboratory (*7,8*). Protein content of the final washed suspension of microsomes was determined by the method of Lowry et al. (*9*) with bovine serum albumin as the standard. Cytochrome P–450 content of the hepatic microsomes was determined by the CO difference method (*10*). Benzphetamine was used as the substrate to determine mixed-function oxidase (MFO) activity. The final concentration of *d*-(+)-benzphetamine in the incubation mixture was 5*mM* and the formaldehyde formed was determined by the method of Nash (*11*).

Statistical Analyses

In each study, we tested for a vinyl chloride effect and for an interaction (in the linear additive sense) between vinyl chloride and either of the other two treatments. The inter-

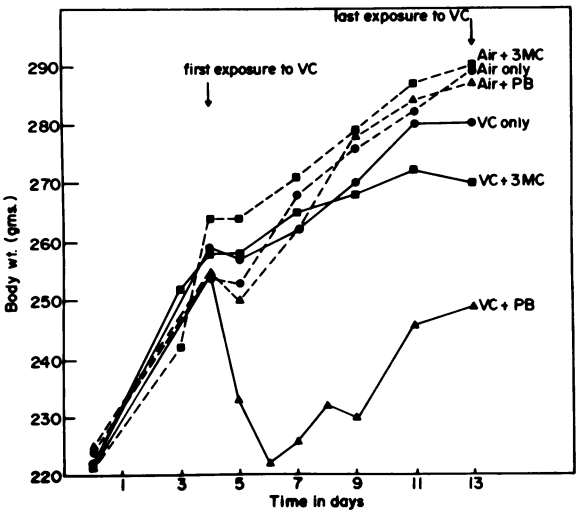

FIGURE 1. Body weights of rats during 10-day exposure to vinyl chloride. The groups treated with both PB and 3-MC and exposed to vinyl chloride are significantly ($p < 0.01$) different from their air-exposed counterparts.

actions were tested for by an F test based on a two-way analysis of variance. The "pure" vinyl chloride effect was tested for by using the Mann-Whitney U test (*12*). In the absence of an interaction according to the F test, the pure vinyl chloride effect was assessed by comparing all vinyl chloride exposed groups to their air-exposed counterparts. When an interaction was observed between vinyl chloride and either 3-MC or PB, that treatment group was omitted from the pooling of the U test for assessment of a pure vinyl chloride effect.

Results

Twenty-four rats were exposed 6 hr/day for 10 consecutive days to an average concentration of 13,500 ppm vinyl chloride. The rats were divided into three groups of eight animals each, one group being treated with 3-MC, one group receiving PB, and one group getting no treatment. Half of the animals in each group were sacrificed on day 11, 18 hr after the last exposure; and the other half of the animals were sacrificed on day 15, 4 days later. Twenty-four "control" rats, treated with 3-MC, phenobarbital or nothing, as above, were exposed to recirculated air 6 hr/day for 10 days. Rats were sacrificed at the same time.

The most marked effect observed was the difference between the body weights of the animals which were exposed to vinyl chloride and treated with PB and the weights of all the other groups (Fig. 1). On the last day of the exposure, both treated groups (with 3-MC or phenobarbital) exposed to vinyl chloride weighed significantly less than their air-exposed counterparts. The animals exposed only to vinyl chloride weighed less than their parallel (air exposed) controls but the difference was not statistically significant (using a nonparametric U test).

Table 1 summarizes the results of tests on animals sacrificed on day 11. As expected, phenobarbital treatment alone causes an increase in liver weight and a 3.5-fold increase in the hepatic microsomal MFO activity. The only effect of 3-MC treatment is a decrease in the hepatic microsomal MFO activity when compared to control animals. Vinyl chloride exposure causes a depression in body weight, liver weight, and kidney weight ($p < 0.05$). There is a suggestion of a diminution in benzphetamine-*N*-demethylase activity caused by exposure to vinyl chloride ($p < 0.065$). There is no indication of an interaction between vinyl chloride and phenobarbital treatments, and the only interaction observed between vinyl chloride and 3-MC treatments was a less than additive effect on the hepatic microsomal cytochrome P-450 concentration.

Four days later (Table 2), the increases in hepatic microsomal MFO activity caused by PB treatment alone were diminished, and the 3-MC-treated animals had increased liver weights. Vinyl chloride given alone causes a decrease in hepatic microsomal benzphetamine-*N*-demethylase activity ($p < 0.05$) and cytochrome P-450 concentration ($p < 0.05$). There is an interaction between vinyl chloride and both PB and 3-MC treatments on the kidney weights, the effect again being less than additive. The interaction between 3-MC and vinyl chloride treatments on hepatic microsomal cytochrome P-450, seen at day 11, is also seen again at day 15.

Morphological changes resulting from exposure of rats to vinyl chloride were not observed in heart, spleen, kidneys, or lungs from any of the animals sacrificed on the day after 10 daily exposures to vinyl chloride. Only the livers of animals treated with phenobarbital

Table 1. Ten consecutive exposures to VC—13,500 ppm; (A) sacrificed on day 11.

Animal treatment[a]	Parameter	Exposed to[b] VC	Exposed to[b] Air
Phenobarbital	Body weight, g	239.0 ± 7.0	284.0 ±9.0
	Liver weight, g	14.1 ± 1.0	16.6 ±0.7
	Kidney weight, g	2.20± 0.10	2.40±0.07
	Benzphetamine metabolism, nmole/mg-min[c]	21.3 ± 1.9	22.8 ±1.0
	Cytochrome P-450 content, nmole/mg[d]	1.5 ± 0.3	2.0 ±0.2
3-MC	Body weight, g	255.0 ± 9.0	293.0 ±8.0
	Liver weight, g	12.5 ± 0.6	14.4 ±0.3
	Kidney weight, g	1.91± 0.07	2.15±0.12
	Benzphetamine metabolism, nmole/mg-min[c]	4.4 ± 0.1	5.1 ±0.2
	Cytochrome P-448 content, nmole/mg[d]	1.5 ± 0.1	1.9 ±0.1
No treatment	Body weight, g	285.0 ±12.0	296.0 ±9.0
	Liver weight, g	12.2 ± 0.7	12.4 ±0.6
	Kidney weight, g	2.34± 0.05	2.37±0.08
	Benzphetamine metabolism, nmole/mg-min[c]	6.5 ± 0.3	6.3 ±1.0
	Cytochrome P-450 content, nmole/mg[d]	0.9 ± 0.1	1.0 ±0.1

[a] Phenobarbital administered in drinking water (1 mg/ml) for 3 days prior to vinyl chloride exposure and continued during exposure. 3-MC injected IP (15 mg/kg) for 2 days prior to first exposure and continued during exposure.
[b] Data reported as mean±S.E.M. ($N=4$). Vinyl chloride caused a significant depression ($p<0.05$) in the liver weight, kidney weight, heart weight (data not shown), and body weight. Vinyl chloride and 3-MC interact in a less than additive fashion on hepatic microsomal cytochrome P-450 concentration.
[c] The units of benzphetamine metabolism are nmoles formaldehyde formed per minute per mg microsomal protein.
[d] Cytochrome concentrations are given as nmoles/mg microsomal protein.

Table 2. Ten consecutive exposures to VC—13,500 ppm; (B) sacrificed on day 15.

Animal treatment[a]	Parameter	Exposed to[b] VC	Exposed to[b] Air
Phenobarbital	Body weight, g	296.0 ±14.0	323.0 ± 3.0
	Liver weight, g	12.9 ± 0.5	14.6 ± 0.4
	Kidney weight, g	2.25± 0.11	2.54± 0.07
	Benzphetamine metabolism, nmole/mg-min	7.0 ± 0.3	9.0 ± 1.0
	Cytochrome P-450 content, nmole/mg	0.9 ± 0.1	1.0 ± 0.1
3-MC	Body weight, g	308.0 ± 9.0	319.0 ± 7.0
	Liver weight, g	15.6 ± 1.1	15.5 ± 0.5
	Kidney weight, g	2.25± 0.06	2.47± 0.09
	Benzphetamine metabolism, nmole/mg-min	4.2 ± 0.2	4.1 ± 0.5
	Cytochrome P-448 content, nmole/mg	1.3 ± 0.1	1.8 ± 0.1
No treatment	Body weight, g	314.0 ±10.0	312.0 ±12.0
	Liver weight, g	11.2 ± 0.2	11.2 ± 0.7
	Kidney weight, g	2.44± 0.08	2.27± 0.09
	Benzphetamine metabolism, nmole/mg-min	5.5 ± 0.3	6.5 ± 0.2
	Cytochrome P-450 content, nmole/mg	0.8 ± 0.04	1.0 ± 0.04

[a] Phenobarbital administered in drinking water (1 mg/ml) for 3 days prior to vinyl chloride exposure and continued during exposure. 3-MC injected IP (15 mg/kg) for 2 days prior to first exposure and continued during exposure.
[b] Data reported as mean±S.E.M. ($N=4$). Vinyl chloride caused a significant ($p<0.05$) decrease in benzphetamine *N*-demethylase activity and cytochrome P-450 concentration and an increase in kidney weight. There was evidence of a significant (less than additive) interaction between vinyl chloride and both phenobarbital and 3-MC on kidney weight and between 3-MC and vinyl chloride on heart weight (data not shown) and cytochrome P-450 concentration.

showed any changes from normal. Treatment with phenobarbital alone caused a slight swelling of the hepatocytes. The livers of animals exposed to both PB and vinyl chloride showed marked differences from controls. In two of the four animals exposed to both vinyl chloride and phenobarbital, there was swelling of hepatocytes, and in the other two there were marked vacuolizations and necrosis around the central veins (Fig. 2). Most of these hepatic

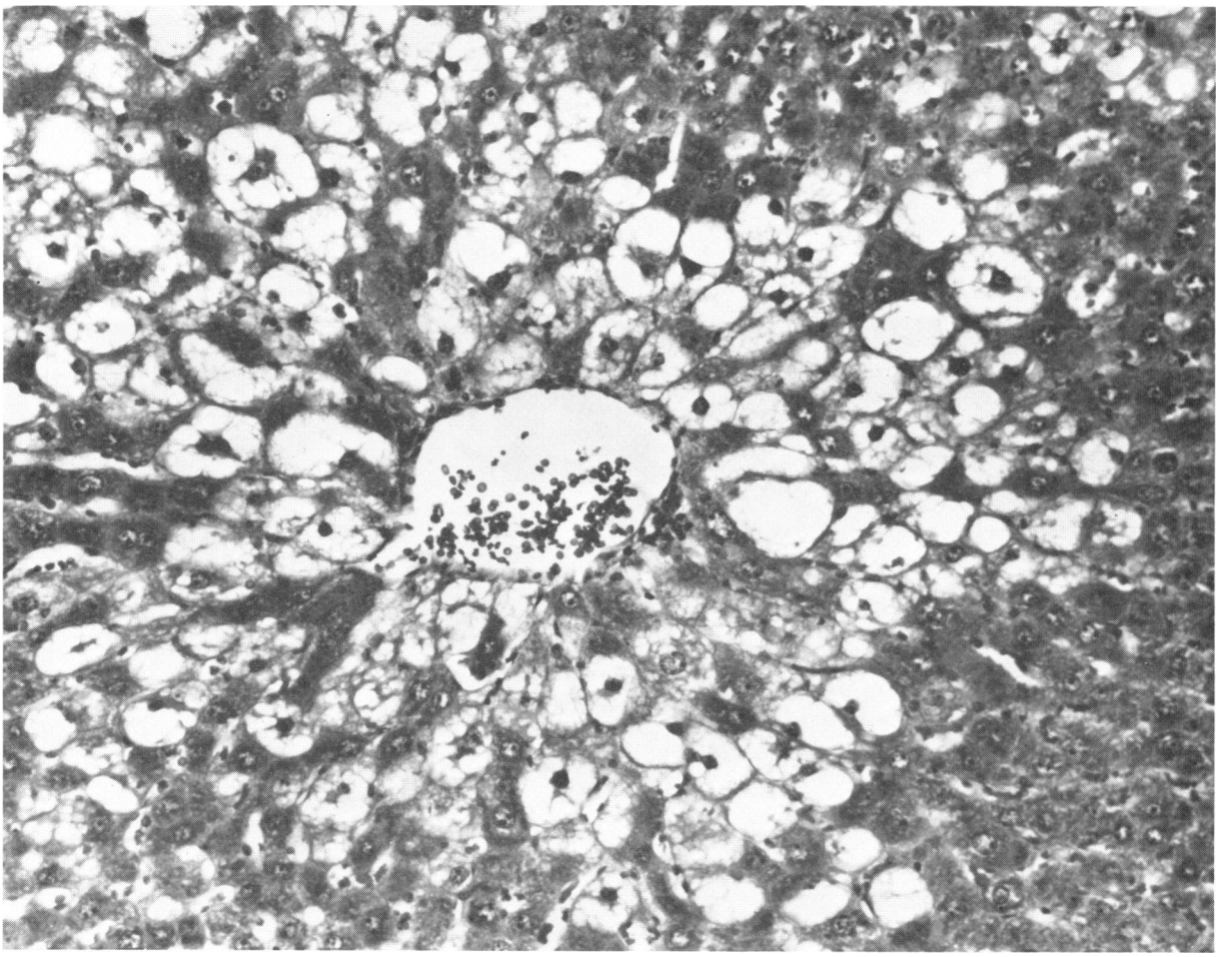

FIGURE 2. Photomicrograph of liver from a rat treated with PB and exposed to vinyl chloride for 10 days and sacrificed on day 11. Note the swelling, vacuolization and necrosis of hepatocytes around the central vein. H & E stain; ×67.

Table 3. Two consecutive exposures to VC—17,300 ppm; sacrificed on day 3.

Animal treatment[a]	Parameter	Exposed to[b]	
		VC	Air
Phenobarbital	Body weight, g	234.0 ±17.0	263.0 ±13.0
	Liver weight, g	14.8 ± 1.3	17.3 ± 1.4
	Kidney weight, g	2.23± 0.15	2.47± 0.21
	Benzphetamine metabolism, nmole/mg-min	15.3 ± 0.3	19.1 ± 1.2
	Cytochrome P–450 content, nmole/mg	1.7 ± 0.7	2.3 ± 0.5
No treatment	Body weight, g	253.0 ±13.0	257.0 ± 8.0
	Liver weight, g	12.6 ± 0.8	12.4 ± 3.4
	Kidney weight, g	2.38± 0.11	2.28± 0.08
	Benzphetamine metabolism, nmole/mg-min	5.2 ± 1.1	7.0 ± 0.5
	Cytochrome P–450 content, nmole/mg	0.7 ± 0.1	0.9 ± 0.1

[a] Phenobarbital administered in drinking water (1 mg/ml) for 3 days prior to vinyl chloride exposure and continued during exposure.

[b] Data reported as mean±S.E.M. ($N=4$). Vinyl chloride caused a depression in benzphetamine N-demethylase activity ($p<0.05$) and cytochrome P–450 concentration ($p<0.06$).

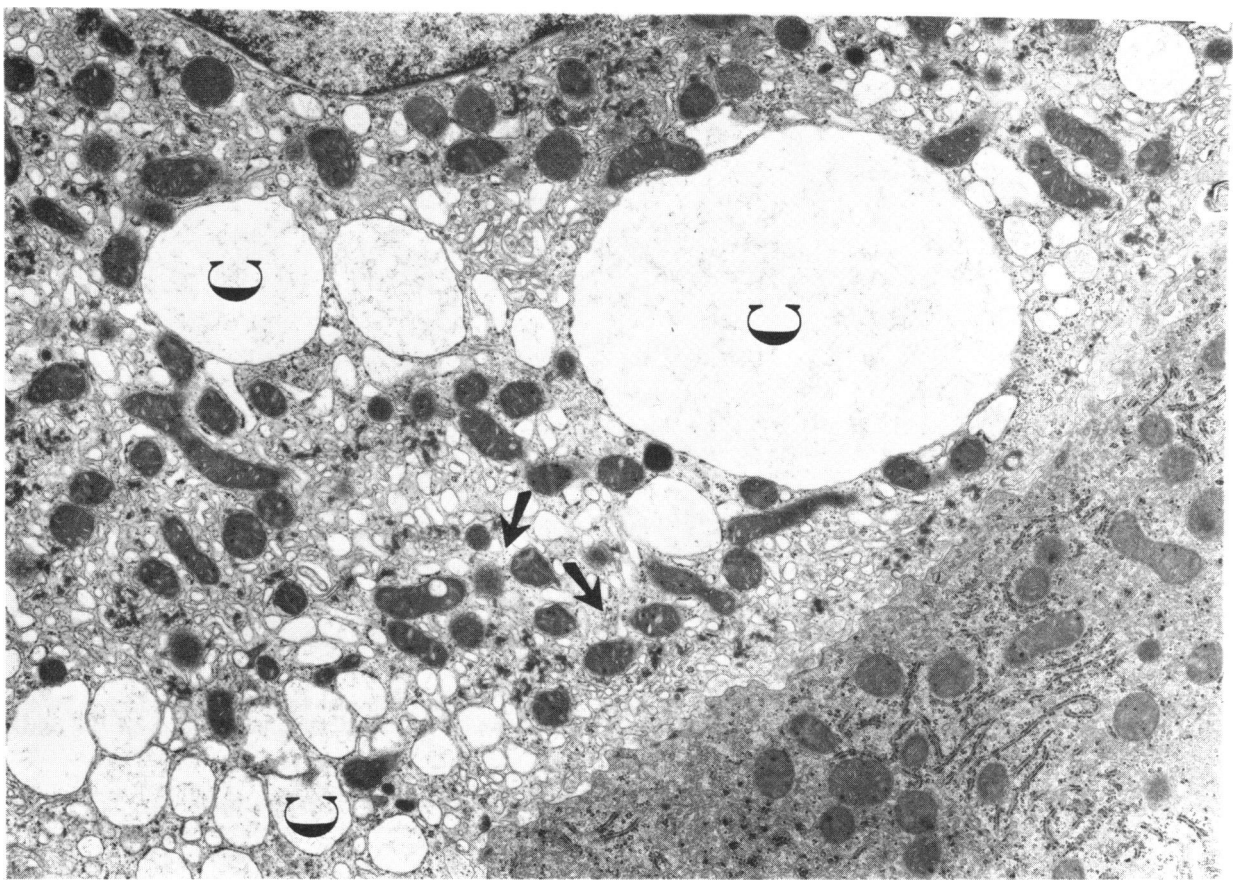

FIGURE 3. Electron micrograph of liver parenchymal cells from a rat treated with PB and exposed to vinyl chloride for 10 days and sacrificed on day 11. Note cell with scattered free ribosomes (arrows) and dilated cisternae (c) which contain proteinaceous material. Compare with adjacent cell of normal morphologic appearance. ×11,234.

changes had diminished by day 15 with only some slight swelling noted in hepatocytes from animals treated with PB and exposed to vinyl chloride. Electron micrographs of liver from these animals also showed changes in the PB-treated, vinyl chloride-exposed animals as compared with controls. Certain hepatocytes from the vinyl chloride- and phenobarbital-treated group contained scattered free ribosomes and dilated cisternae containing proteinaceous material (Fig. 3).

The marked weight changes on the first two days of vinyl chloride exposure of phenobarbital-treated rats prompted a second study. Eight rats, four with PB in their drinking water and four without, were exposed for two

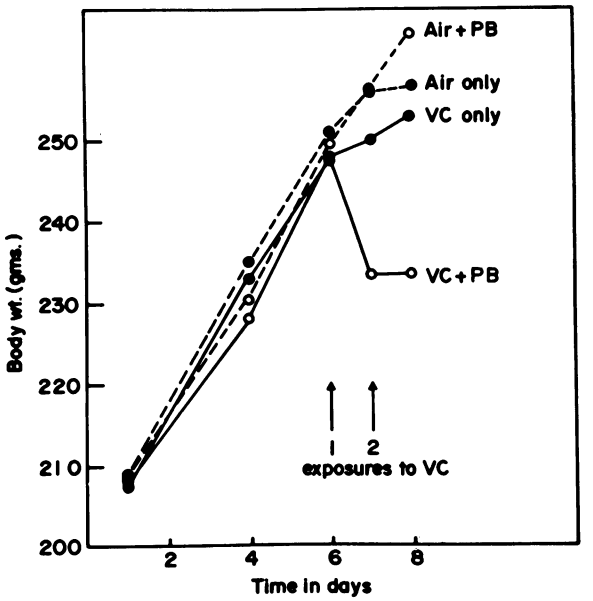

FIGURE 4. Body weights of rats during 2-day exposure to vinyl chloride. The group treated with PB and vinyl chloride gained significantly less weight than any other group during the exposure period.

consecutive days, 6 hr/day, to vinyl chloride at an average concentration of 17,300 ppm. A similar group of eight rats, four with PB in their drinking water and four without, were exposed to air. All 16 animals were sacrificed on the third morning. The weight gain of these animals is shown in Figure 4. When overall weight change was investigated, an indication of a synergistic (i.e., greater than additive) interaction between vinyl chloride and PB was observed ($p = 0.06$). Vinyl chloride in the presence of PB treatment caused a depressed level of weight gain relative to its effect in the absence of PB. Individual daily weight changes were examined and showed that the only indication of an interaction between vinyl chloride and phenobarbital occurred between the first and second exposures where the weight change of PB-treated animals exposed to vinyl chloride is significantly less than that in any of the other three groups ($p < 0.028$).

The other results (Table 3) of our second study were qualitatively similar to our first study with one exception. The vinyl chloride exposures decreased the MFO activity in both the PB-treated and untreated groups. At all three sacrifice times, the increase in liver weight caused by PB appears to be partially reversed by adding vinyl chloride.

Morphologic changes in this second experimental group of rats were not uniform. In both vinyl chloride-exposed groups, one of four rats had marked changes. In the PB plus vinyl chloride group, one rat had marked centrilobular necrosis with vacuolization of hepatocytes and fatty infiltration. One of the four animals exposed to vinyl chloride only had an aggregation of a few hepatocytes which were larger than their surrounding cells and had hyperchromic nuclei. The lungs of this animal also had changes characterized by alveolar epithelialization in one area only. The ultrastructural changes in liver of animals in the group exposed to both phenobarbital and vinyl chloride were similar to those seen after 10 exposures.

Discussion

These studies confirm the results reported by Reynolds et al. (*13*), who described similar findings after rats treated with phenobarbital were given a single exposure to 50,000 ppm vinyl chloride. The lesions we found after phenobarbital-treated rats had received ten exposures to 13,500 ppm vinyl chloride were similar although perhaps not quite as severe as those seen by Reynolds after a single exposure to a higher concentration of vinyl chloride. In order for vinyl chloride to produce an effect in both studies (at the concentrations investigated), it was necessary to treat the animals with phenobarbital.

The results also raise the question of individual susceptibility or sensivity to vinyl chloride. The response of rats in both our studies was not uniform. After 10 exposures the livers of animals treated with phenobarbital and exposed to vinyl chloride showed a wide variation of response, from slight swelling of hepatocytes in one animal to vacuolization and necrosis in another. In the second study, one of four animals in each vinyl chloride-exposed group responded to a greater degree than the others.

Other than the liver changes, the only marked effect of vinyl chloride was in the weight gain of animals treated with phenobarbital and also exposed to vinyl chloride. In both our studies, the weight gain was particularly depressed on the second day of the vinyl chloride exposures.

Finally, while we have shown changes resulting from exposure to a concentration of vinyl chloride and phenobarbital, we have not shown that these changes are preneoplastic. Further

experiments are necessary to understand the relationship between the liver changes seen here and the carcinogenicity of vinyl chloride.

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